ADDIS ABABA UNIVERSITY
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MOLECULAR IDENTIFICATION FOR SIX VIRULENT GENES OF *ESCHERICHIA COLI* ISOLATED FROM DIARRHEIC CALVES AND THEIR RESISTANCE PROFILE TO ANTIMICROBIALS IN SELECTED TOWNS OF SOUTH WOLLO ADMINISTRATIVE ZONE, AMHARA, ETHIOPIA.

FENTAW HUSSEN

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SCHOOL OF GRADUATE STUDIES

This is to certify that the dissertation prepared by **Fentaw Hussen**, titled: **Molecular identification for six virulent genes of *Escherichia coli* isolated from diarrheic calves and their resistance profile to antimicrobials in selected towns of South Wollo Administrative Zone, Amhara, Ethiopia, 2017/18** and Submitted in partial fulfillment of the requirements for the degree "Master of Science in Biochemistry" in the department of medical Biochemistry complies with regulations of the university and meets the accepted standards with respect to originality and quality.

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ABBREVIATIONS/ACRONYMS

aEPEC	Atypical enteropathogenic *Escherichia coli*
BFP	Bundle forming pilli
bp	base pair
dNTP	Deoxy ribonucleotide triphosphate
EAEC	Entero aggregative *Escherichia coli*
EHEC	Entero hemorrhagic *Escherichia coli*
EPEC	Entero pathogenic *Escherichia coli*
Gb3	Globotriaosyl ceramide
HGT	Horizontal gene transfer
HUS	Human uremic syndrome
IMViC	Indole, Methyl red, vocusproskaure and citrate
kDa	Killo dalton
LEE	Locus of enterocyte effacement
MDR	Multi drug resistance
PAI	Pathogenecity island
PCR	Polymerized chain reaction
STEC	Shigatoxigenic *Escherichia coli*
tEPEC	Typical enteropathogenic *Escherichia coli*
UPEC	uropathogenic *Escherichia coli*

ABSTRACT

Background: *Escherichia coli* are normal flora of the gastrointestinal tract frequently used as model for various research questions. Potential gain and loss of virulent genes enable them to become pathogenic and causes calf diarrhea while beneficial ones subsidize vitamin K_2. These virulent genes are found on plasmids, genomes or integrated on bacteriophages and horizontal gene transfer plays in its epidemiology.

Objective: To identify virulence genes informative for four pathogenic strains of *E. coli and study* their antibiotic resistance patterns to eleven antimicrobials agents.

Methodology: The study was conducted in Kombolcha, Dessie and Haik areas. Purposive sampling was used to collect 123 fecal samples from 53 farms of all sites. *E. coli* isolates were identified based on their green metallic sheen appearance on EMB agar, their characteristic reactions on MacConkey agar and IMViC (+ + - -) biochemical tests. Antimicrobial susceptibility testing of the isolates was carried out using agar disc diffusion method on Muller Hinton agar following standard procedures. Genomic DNA was extracted from the isolates using boiling method for strain identification using PCR.

Results: One hundred (81.3%) pure isolates of *E. coli* were harvested, of which, 23 (29.9%) isolates carried one or more of the tested virulent genes. *stx_1* 14(18.2%), stx_2 7(9.1%), *eae* 9(11.7%), *hlyA* 1(1.3%) were the detected virulent genes with their respective frequency. However, no isolates were detected for *bfpA* and *aatA* genes. Virulent genes were detected in 2-4 weeks aged calves at significantly higher order ($P \leq 0.05$). Calves housed with their dam and those given supplement feed were significantly associated with detected genes ($P \leq 0.05$). From samples processed using PCR, 14 (18.2%) were confirmed as STEC strain, 5 (6.5%) of EHEC and 4 (5.2%) of EPEC. Most isolates were resistant to sulfonamide, clindamycine, ampicillin, Oxytetracyclne, trimethoprime and streptomycin and sensitive to nitrfurantoin, gentamycin, neomycin, clorampfenicol and ciprofloxacine.

Conclusion: Zoonotic STEC and EHEC strains are collectively found highest and implies reservoir of calves to both farm animals and humans. Poor farm hygiene and frequent use of limited antibiotics was a problem observed. As a consequence, farms hygiene, more spatial and temporal distribution of *E. coli* should be concerned. Additionally, Antibiotics should be alternatively used to avoid resistance.

Keywords: Antimicrobials, Calf, *Escherichia coli*, Resistance Strains, Virulent Genes.

1. INTRODUCTION

1.1. Background

The isolation and characterization of slender short rods of *Escherichia coli* was first reported by Theodore Escherich, that was later known as a harmless commensal of the gastrointestinal tract in warm-blooded animals(Gomes *et al.*, 2016). Sequential investigations confirmed that *Escherichia coli* is a gram-negative, rod-shaped, flagellated, non-sporulating, facultative anaerobe and nonmotile or motile bacterium of the family enterobacteriaceae(Shahrani *et al.*, 2014).

Most *Escherichia coli* strains are harmless and form part of the normal flora of the gastrointestinal tract. Beneficial *Escherichia coli* can produce vitamin K2 for the host while pathogenic *E. coli* causes a broad range of calf diarrhea(Umpierrez *et al.*, 2017) and can extend to extra intestinal sites(Gebregiorgis and Tessema, 2016; Picco *et al.*, 2015). Now a day's several studies have addressed the higher distribution of pathogenic *E. coli* strains in infectious calf diarrhea that are classically grouped into enterohemorrhagic (EHEC), enterotoxigenic (ETEC), necrotoxigenic (NTEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and attaching and effacing *E. coli* (AEEC) pathotypes (Shahrani *et al.*, 2014; Croxen *et al.*, 2013).

The diarheagenic nature of *E. coli* is due to its potential gain and loss of virulent genes that enable it to become highly diverse and adapted pathogen(Croxen *et al.*, 2013). The genes encoding *E. coli* virulence factors are located either on plasmids, on large genome regions called pathogenicity islands (10 to 200 kb), or on integrated bacteriophages(Lambertini *et al.*, 2015). Inter-transfer of DNA between prokaryotic hosts could also be achieved through mechanisms like conjugation, transformation and transduction for horizontal gene transfer. Expression of fimbrial (pilli) antigens that allow to adhere and colonize the luminal surface of the small intestine and elaboration of enterotoxins that influence intestinal secretion of fluids are the two prominent pathogenic role of virulent gene products of this bacterium(Gebregiorgis and Tessema, 2016).

Insertion of virulence genes to the bacterium can cause moderate to severe scours and even may lead to death. For this reason *Escherichia coli* today is the most commonly known bacterial cause of diarrhea in young animals that have both economic and pervasive concern in veterinary industry all around the world(Shahrani *et al.*, 2014). In contrast, the apparent resistance to *E. coli* infection while

aging has been attributed to the loss of specific receptors with age or development of immunity(Croxen *et al.*, 2013).

Diarrheagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were developed(Nataro and Kaper, 1998). Although different approaches attempted to diagnose pathogenic *E. coli*, polymerase chain reaction (PCR) is classically a sole and confirmatory nucleic acid-based method for detection of virulent genes. PCR exploits the thermo cyclic enzymatic amplification of specific DNA sequences of the target gene using a pair of oligo nucleotide primers(Cho and Yoon, 2014).

Diarrheagenic *E. coli* often require antimicrobial intervention as well as fluid and electrolyte replacement. However, increase in evidence for antibiotic-resistant strains of this bacterium to wide range of antibiotics cause longer and more severe illnesses or neonatal death than their antibiotic-susceptible counterparts(Shahrani *et al.*, 2014). Data on the distribution and severity of virulence genes and the antimicrobial resistance properties of pathogenic *E. coli* strains isolated from diarrheic calves is scarce in Ethiopia.

1.2. Statement of the problem

There is an increase in the use of exotic dairy cattle and their crosses in order to enhance milk production. However, since exotic cattle are less tolerant to local diseases, the dairy production is facing a great challenge to higher morbidity and mortality in dairy cows and their offspring(Asmare and Kiros, 2016). Intensive animal breeding systems also have recently increased the transmission of infectious diseases(Umpierrez *et al.*, 2017). Consequently, high mortality rate associated with various infectious agents in calves under 3-weeks-old and up to 3-months-old has been reported(Cho and Yoon, 2014). *E. coli* is the most important cause of bacterial scours in calves(Yeshiwas and Fentahun, 2017) and poses a significant treatment cost, genetic loss and impaired future performance. Although a study based on culture and biotyping has been carried out in Kombolcha previously, *E. coli* has never been studied in adjacent cosmopolitan areas of Dessie and Haik. Furthermore, molecular based detection of pathogenic strains has never been studied in all the three sites. The involvement of virulence genes of *E. coli* in calf diarrhea is being studied in Ethiopia since recent years.

1.3. Significance of the study

Identification of Enteric Pathogenic *Escherichia coli* added information on the other infectious agents that cause diarrhea in calves. The result is believed to be an excellent input to the local farm owners and concerned professionals to its effective treatment based on the indicated level of resistance to locally administering antimicrobial agents. Molecular identification of these pathogens explores prompt distribution of pathogenic strains, together with their abundance, in line with the investigated risk factors. Identification of dominant pathogenic strains is an important data that can be used to design appropriate diagnostic and vaccine strategies.

1.4. Objectives

1.4.1. General Objective

- To identify virulent genes indicative of pathogenic strains of *Escherichia coli* in diarrheic calve and the resistance pattern of isolates to antimicrobials at Dessie, Kombolcha and Haik towns of Amhara Regional State, South Wollo, Ethiopia.

1.4.2. Specific objectives

- To isolate and identify *Escherichia coli* from diarrheic calves using standard bacteriological methods.
- To determine the antibiotic susceptibility patterns of *E. coli* isolates
- To identify the pathogenic *E. coli* strains involved in calf diarrhea using PCR
- To determine predisposing risk factors associated with presence of *E. coli*, in general, and pathogenic *E. coli* in particular.

1.5. Hypothesis

Null hypothesis (H_o): There is no difference in the occurrence of virulent genes and strains of *Escherichia coli* and the factors of interest in the diarrheic calves.

Alternative hypothesis (H_1): There is variation in frequency among the virulent genes, and strain, and risk factors.

2. LITERATURE REVIEW

2.1.Enterovirulent *E. coli*

Beyond single primary pathogen or co-infection, calf diarrhea can also be caused by other factors such as nutrition, hygienic conditions and environmentally related issues(Hashish *et al.*, 2016). Until immunity is compromised and the epithelial integrity is breached off *E. coli* remains and coexist as part of the gut micro biota(Umpierrez *et al.*, 2016).

The versatility of the *E. coli* genome is conferred mainly by virulence-related plasmids and chromosomal pathogenicity islands(Nataro and Kaper, 1998). There is a clear difference in genome size of commensals and pathogenic *E. coli* by a million base pairs where this extra genetic content can contain virulence and fitness genes. *E. coli* have both core genome and flexible gene pool and it has been reported that pathogenic ability is largely afforded by the flexible gene pool through the gain and loss of genetic material(Kaas *et al.*, 2012). All six gene of *E. coli* described in this paper have been shown to be carried either in LEE or plasmid.

DNA can be moved between prokaryotic hosts and provides either new trait for fitness advantage or virulence. This is achieved through mechanisms such as conjugation, transformation, and transduction exploiting factors encoded by mobile genetic elements, such as transposons, insertion sequences, bacteriophages, and plasmids resulting in horizontal gene transfer(Frost *et al.*, 2005).

Even though genome sequences of pathogenic *E. coli* are reportedly being completed, the genetic diversity of this organism is believed to be very vast and defining it based on a small set of features is challenging. This is because many of these defining genes may not be restricted to a particular pathotype but is still informative to consider the main pathotypes as a framework to an overview of enteric *E. coli* (Croxen *et al.*, 2013). Typical and atypical enteropathogenic *E. coli* (tEPEC/aEPEC), Enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and Enteroinvasive *E. coli*(EIEC) are responsible pathogenic *E. coli* strain in both young animal and children diarrhea worldwide (Gomes *et al.*, 2016) where genes describing the first three are gone through in this particular work.

2.2.Virulent factors of *Escherichia coli*

2.2.1. Intimin gene (eae) and its product

The gene *eae* is one of the virulence genes of *E. coli* responsible to encode a protein called intimin, a 94-kDa outer membrane protein involved in the intimate adherence between bacteria and enterocyte membranes(Croxen *et al.*, 2013; Trabulsi *et al.*, 2002). The gene is located within a 35-kb chromosomal region of EPEC strain, called locus of enterocyte effacement (LEE), cluster of genes comprise 41 genes(Sperandio and Nguyen, 2012; Lacher *et al.*, 2006). This classical group of genes is absent in normal flora of *Escherichia coli* (Nataro and Kaper, 1998). An interaction between intimin and the bacterial translocated intimin receptor (Tir) results intestinal adherence and effacement lesion by subverting actin dynamics within enterocytes(Croxen *et al.*, 2013; Jafari *et al.*, 2012; Gruenheid *et al.*, 2001).

Intimate attachments of EPEC with enterocyte interaction induce diverse signal transduction pathways within host cell and production of many cellular proteins encoded from LEE PAI of *E. coli*. LEE region encodes both core and regulatory genes to produce core and regulatory proteins respectively. One of the core protein encoded from LEE is a T3SS, which secrete protein components of the translocon (EspA, EspB, and EspD) to drive effectors directly into host cells. The regulatory proteins, secreted effectors and their related chaperones also generates from LEE(Croxen *et al.*, 2013).

Shigatoxigenic *E. coli* also secrete non-LEE encoded, *E. coli* secreted protein F-like protein from prophageU (EspFu). This protein activates actin nucleation promoting factor Wiskott-Aldrich syndrome protein (N-WASP) and insulin receptor tyrosine kinase substrate p53 (IRSp53), to regulate actin cytoskeleton reorganization. It results actin accumulation beneath attached bacteria, and gives the area pedestal-like structure(Weiss *et al.*, 2009). Primary interaction of Tir and intimin play a crucial role in intestinal colonization and proceeding of further cascade of signal transduction to cause neonatal calves diarrhea(Sperandio and Nguyen, 2012).

Intimin gene is principally amplified in various works and can be found alone or together with other virulent genes in isolates of *E. coli* giving it EPEC or EHEC. The presence of this gene is therefore informative to cause of calf diarrhea. Lots of works have been documented on this virulent gene from various parts of the world at various times. For instance, in the work of Umpierrez *et al.*,

(2017), PCR amplification of *eae* showed an important component in the prevalence of EHEC and EPEC.

In Iran *E. coli* were isolated from diarrheic calves to detect few virulent genes to which *eae* prevalence was reported to be 2.6% (Badouei *et al.*, 2014). 60.3% (De Moura *et al.*, 2012), 1.3% (Picco *et al.*, 2015), 2.1% (Umpierrez *et al.*, 2017) and 2% (Rehman *et al.*, 2014) from Brazil, Argentina, Uruguay and Kashmir respectively. Additionally, unpublished documents based on recent works in Ethiopia showed frequency of 19.3% in Wolayta Sodo (Amanuel *et al.*, 2018) and 16.4% in Hawassa (Tsedale *et al.*, 2018).

2.2.2. Shiga like toxin genes (stx$_1$ and stx$_2$) and their products

Both human and bovine derived STEC produces two phage encoded potent cytotoxins(Pizarro *et al.*, 2013). These toxic proteins produced can be classified into Shiga toxin one (Stx_1) and two (Stx_2) encoded by stx_1 and stx_2 genes respectively on prophages integrated into the chromosome. stx_1 and stx_2 variants on STEC can be found either alone or in combination and the toxin is released from lysed bacterial cells during the lytic cycle of the phage while bacteria gets stressed. Loss of the *stx*-containing phage has been reported several days after isolation(Croxen *et al.*, 2013).

It is hypothesized that STEC-induced inflammation may provide the toxin an opportunity to breach the epithelial barrier and other study proposed that STEC is able to cross the intestinal epithelium through microfold cells(Mcells) and survive in macrophages, and this may be a way for the Shiga toxin to be released into the bloodstream, where it can target other organs(Etienne-Mesmin *et al.*, 2011). Stx binds to Gb3 on the surface of endothelial cells and is internalized and trafficked through the retrograde pathway from the Golgi apparatus and endoplasmic reticulum (ER) and eventually to the host cell cytoplasm(Tesh, 2012).

Shiga toxin is composed of A and B subunits where B subunit forms a pentamer that binds to globotriaosylceramide-3 (Gb3), where its patho-physiology is specifically mediated in human. The A subunit of this toxin is responsible to cell death where the mechanisms involve several stress pathways in a complex way. This subunit is generally an RNA-glycosidase that removes an adenine from 28S rRNA, thereby inhibiting protein synthesis and causing cell death(Tesh, 2012). Gb3 expressed on human's vascular endothelium to promote much of the patho-physiology associated with Shiga toxin. As a result the toxin binds and absorbed through Gb3 expressed endothelial cells

to the blood stream and disseminated to other organs to cause blood related diseases like hemorrhagic diarrhea and human uremic syndrome (HUS). In addition to this EHEC of human colonizes colon and causes electrolyte imbalances(Sperandio and Nguyen, 2012).

On the other view unlike to humans, toxicity with STEC is asymptomatic in adult ruminants (Sperandio and Nguyen, 2012) and diarheagenic in calves, if the dose of toxin is higher(Kuyucuoglu *et al.,* 2011). Recent outputs indicated for binding difference between Stx1 and Stx2 that might involve more than one glycan(Kolenda *et al.,* 2015). That blood related diseases mentioned above on humans didn't challenge the dairy industry due to lack of vascular expression of Gb3. Although Gb3 receptors are detected in their kidney and brain, Shiga toxin was unable to reach to these organs due to absence of Gb3 in intestinal associated blood vessels, needed for endocytosis and further transportation(Sperandio and Nguyen, 2012). Again in contrast to human shiga toxigenesis, the impervious effects of STEC in catle is due to its tendency of colonizing recto-anal junction (RAJ) of intestine than invading colon(Cobbold *et al.,* 2007; Naylor *et al.,* 2003).

Cattle's cyclic reservoir for this *stx* bearing zoonotically hazardous *E. coli* is supported with three possible reason of successful survival on their alimentary canal. First one is its potential survival in acidic environment of the stomach. This is achieved in short by exploiting glucose repression with bacterial encoded proteins like the cAMP receptor protein (CRP) and the stress response alternative sigma factor (RpoS), presence of activity of glutamate and arginine decarboxylases that increases proton up take there by maintaining pH homeostasis. Secondly, classical release of both LEE encoded and EspFu proteins are utilized for actin reorganization and facilitate its attachment and colonization at recto anal junction(Sperandio and Nguyen, 2012).

Finally, EHEC can produce regulatory factors to both acid resistance and A/E lesion formation. Acyl-homo serine lactones (AHLs), produced by vicinity bacteria, mediated sensing by SdiA (a mechanism termed quorum- sensing) activate the *gad* (acid fitness) genes in the rumen. It renders bacteria resistant to acid in the region, thereby; repressing the LEE genes to prevent colonization within the rumen. No AHLs will be detected by SdiA of EHEC in compartments beyond the rumen, hence, prevent LEE suppression and allow it to colonize the rectoanal junction(Hughes *et al.*, 2010).

Genes for Shiga toxin were the most frequently detected among virulent genes in *E. coli* isolates from diarrheic calves. Likewise mentioned above for *eae, stx* genes are also found either solely or in combination with other virulent genes rendering the isolate EHEC or STEC. In most of the works

stx₁ identified more than its counterpart *stx₂*. *stx₁* and *stx₂* genes were identified at 12.2% and 7.8% respectively(Herrera-Luna *et al.*, 2009). Hashish *et al.* (2016) found *stx₁* and *stx₂* at 86.67% and 26.67%, respectively. stx_1 was also amplified at 41.3% in the work of De Moura *et al.* (2012) in Brazil and 4.8% were found from Uruguay (Umpierrez *et al.*, 2017). Tsedale and her co workers (2018) found 3% *stx₂* and 19.4% of *stx₁* in calves from Hawassa, Ethiopia. Again respective frequencies of *stx₁* and *stx₂* were reported as 35.1% and 22.8% in Wolyta Sodom, Ethiopia by Amanuel *et al.*, (2018). In contrast, a finding found in Iran showed higher *stx₂* (30%) than *stx₁* gene (10%)(Dastmalchi and Ayremlou, 2012). Likewise, STEC harboring *stx₂* isolate was significantly more (53.42%) than stx_1 carried STEC (10.27%) (Tahamtan *et al.*, 2010). Works have mentioned that Stx_2 toxin (encoded by *stx₂*) has been found to be approximately 400 times more toxic than did Stx_1 (Tahamtan *et al.*, 2010).

In relation to the *stx* genes, EHEC and STEC strains of pathogenic *E. coli* can be derived. In most of studies EHEC considered as subset of STEC consequently, result independently might not be found in clear way. For instance, study in Vietnam on diarrheic calves, 51.3% of their samples were positive for all *stx* genes to whom 6.7% are found mixed with *eae* and categorized as EHEC (Nguyen *et al.*, 2011). EHEC works of Umpierrez *et al.*, (2017) and Badouei *et al.*, (2014) seen to with equal prevalence of 0.7%. On the other side, STEC found from Dastmalchi and Ayremlou, (2012), Umpierrez *et al.*, (2017) and Badouei *et al.*, *(*2014) revealed to have respective prevalence of 11.8%, 0.83% and 3.4% and higher prevalence was reported as much as 44.6% (Nguyen *et al.*, 2011) and 38.6% (Amanuel *et al.*, 2018). Beyond samples from diarrheic calves, sample from apparently healthy cattle in Bangladesh were also found 43.33% of STEC prevalence (Hassan *et al.*, 2017).

2.2.3. aatA gene and its product

Gene *aatA* is one of the virulent genes of enteroaggregative *E. coli* (EAEC), a pathogenic strain in the digestive tract that causes a severe and stable diarrhea. Like wise to other virulence genes of this strain, *aatA* gene also mostly located on the 55,989 bp plasmid. This gene produces a membrane protein necessary for translocation of pathogenic proteins(Nazemi *et al.*, 2011). The *aatA* gene in EAEC forms part of a plasmid encoded locus also under the control of *aggR* coding for an ABC transporter complex that channels the virulence factor dispersin out of the bacterial cell commonly associated with UPEC but also found in diarrheagenic *E. coli* (Aijuka *et al.*, 2018).

Sufficient information explaining about *aatA* from diarrheic calves was not found except recent works confirmed in three isolates (4.5%) from Hawassa, Ethiopia (Tsedale *et al.*, 2018). However, *aatA* is found in various works of *E. coli* isolated from samples other than calf diarrhea. For instance 3.4% from human food sources and irrigation water (Aijuka *et al.*, 2018). 12.5% from malnourished stool sample of children(Havt *et al.*, 2017) and 35.4% from UPEC in human(Nazemi *et al.*, 2011).

2.2.4. hlyA gene and HemolysinA

Hemolysin is a pore-forming toxin encoded by plasmid bearing hlyA gene of *E. coli* that has a potential to lyse erythrocytes(Croxen *et al.*, 2013). Although the role of entero-haemolysin in an intestinal disease is unclear, it has been suggested that entero-haemolysins may enhance the effects of Shiga toxins(Herrera-Luna *et al.*, 2009). The toxin is produced by many strains of *E. coli*(Lorenz *et al.*, 2013) that can be isolated from both healthy/diseased animals and humans and their specific physiological or pathophysiological role remains unclear(Kolenda *et al.*, 2015). These toxins have been shown to be involved in endothelial cytotoxicity(Aldick *et al.*, 2007), cargo with outer membrane vesicles(Aldick *et al.*, 2009) and was shown to be inactivated by another STEC virulence factor, EspP(Brockmeyer *et al.*, 2011).

This virulent gene is found with STEC/EHEC in most of the assessed documents. On the other hand 7% of the isolates carried hlyA gene of which 3.5% were from STEC strain and the remaining 3.5% from EHEC. This gene was also found in 2.5% (Taghadosi *et al.*, 2018) and 60% in Iran(Dastmalchi and Ayremlou, 2012). *E. coli* isolates with this genetic profile (*stx_1/stx_2/hlyA* combination*)* in calves from Iran was reported to be the most prevalent in calves(Dastmalchi and Ayremlou, 2012).

2.2.5. bfpA gene and Bundle forming pilli(bfp)

Bundle forming pilli gene is the one among the cluster of genes encompassed on, a large ~80 kb, EAF plasmid that is responsible to encode a protein called bundle forming pili (BFP)(Cleary *et al.*, 2004; Trabulsi *et al.*, 2002). BFP are involved in bacteria–bacteria interaction and micro colony formation. Thus, it promotes their stabilization and produces a localized adherence (LA) pattern in the form of compact three-dimensional micro colonies that can be seen within 3 h of infection(Cleary *et al.*, 2004). This plasmid encoded protein is the predominant factor that mediates initial attachment of tEPEC to the surface of the host intestinal epithelium(Croxen *et al.*, 2013).

Published documents and relevant alternatives were assessed on status of *bfpA* for typical EPEC and from diarrheic calves. However, little was found about *bfpA* to which no isolates found carried it from the works of Sweden(De Verdier *et al.*, 2012), Wolayta Sodo, Ethiopia (Amanuel *et al.*, 2018) and Hawassa (Tsedale *et al.*, 2018). *hlyA, bfpA* and *aatA* are plasmid genes. Therefore, use of plasmid genes as indicative for pathogenic *E. coli* prevalence have its own limitation such as, its variable gene content, it may lost on sub-culture and the plasmid may transfer and be detected in entirely unrelated bacteria which are not actually able to cause diarrhea(Chattaway *et al.*, 2011).

2.3.Antimicrobial resistance profile of *E. coli*

Antimicrobial resistance has been recognized as an emerging worldwide problem in both human and veterinary medicine(Rasheed *et al.*, 2014). *E. coli* has become resistant to many antimicrobials through the acquisition of mobile drug resistance genes and the incidence posed by this strain has been increasing(Hossain *et al.*, 2013). Un responsiveness to multiple drugs is frequently isolated from the commensal gut flora of food animals and possible transfer of resistance elements to zoonotic pathogens within the gut has serious implications for public health. Possibility of resistance achieved with both horizontally, through the movement of mobile genetic elements, and vertically, through proliferation and subsequent dissemination of resistant bacterial strains(Hoyle *et al.*, 2005).

Extensive and uncontrolled treatment of farm animals plays a major role in an emergence of antimicrobial resistant strains and has become a serious issue this day. Resistance develops due to mechanisms incorporating acquisition of gene-encoding enzymes (*e.g.* β-lactamases), increased activity of efflux pumps, acquisition of several genes encoding bacterial cell walls lacking binding sites for antimicrobials, and mutations leading to decreased permeability. If those resistance genes are on plasmids, it can be transferred rapidly among a number of bacterial species(Chirila *et al.*, 2017).

Because resistance genes can be found in clusters, the recipient can obtain it together, and dissemination of multi-drug resistance will develop through the horizontal genes transfer. In addition, these resistance genes may be transferred by conjugation, transformation, or transduction(Chirila *et al.*, 2017). As a consequence presence of resistance traits and horizontal gene transfer due to indiscriminate use of antibiotics may favoring inter-species resistance transmission

and hindering methods to treat bacterial infections and generates important public health issue(Umpierrez *et al.*, 2017).

Higher resistance of *E. coli* isolates has been reported this day for various antimicrobial agents. The descending resistance level of streptomycin (98.25%), tetracycline (98.09%), sulfonamides (90.31%), gentamycin (79.68%), chloramphenicol (73.8%), ampicillin (71.11%), trimethoprim (62.22%) ciprofloxacin (60.31%) and lowest resistance for nitrofurantoin (23.96%) was shown for *E. coli* isolates in Iran (Shahrani *e al.,* 2014).

E. coli isolates carried virulence gene/s had also shown resistance to one or more antibiotics. Evidence based increment of multidrug resistance (MDR) with time was released in various research outputs. For instance, between years of 1980s and 2000s *E. coli* isolates have shown gradual increase of resistance for tetracycline, sulfonamide and streptomycin in young animals. Existence of genes conferring selective advantage to colonize the intestinal lumen of calves for few antimicrobials has also been mentioned to have a linkage between resistance genes as an additional factor (De Verdier *et al.,* 2012).

2.4.Zoonotic Importance

Due to changes in our daily eating habit and other routine life activities involving the consumption of contaminated food have become a major morbidity and mortality cause around the world(Pizarro *et al.*, 2013). Many human and animal EPEC species are clonally related sharing many virulence properties hence potentially a threat to human health(Moura *et al.*, 2009). Virulence machinery for potential sources of EPEC that are pathogenic to humans can be possessed by cattle and transmission occurs via food chain, direct contacts with their feces and their environment may represent an increased risk factor for human disease(Rehman *et al.*, 2014).

Enterohemorrhaegic *E. coli* is the most importantly incriminated pathogenic *E. coli* in zoonosis and approximately 75% of out breaks in human are linked to the consumption of contaminated bovine derived products. More than 380 different STEC serotypes have been isolated from humans and animals, but only a small number of serotypes are linked to human disease. Serotype O157:H7 is reported in various research output as the major source of *E. coli* food poisoning out breaks in human characterized by abdominal cramps, bloody diarrhea and HUS(Sperandio and Nguyen, 2012).

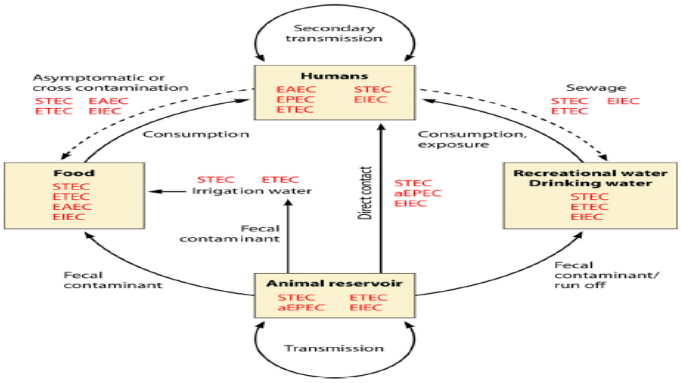

Figure 1: General over view of potential reservoir and modes of transmission for pathogenic *E. coli* (adopted from Croxen *et al.*, 2013).

Various animals bear Pathogenic *E. coli* strains and their feces can contaminate human food, water and environment. Human can encounter it through un cooked or poorly cooked food, drinking/recreational water and other mean. Or secondary transmission can occur between humans accordingly.

Ruminants such as both dairy cattle and beef cattle are widely known to be major reservoirs for pathogenic STEC, and exposure to their fecal matter represents an important source of human illness(Croxen *et al.*, 2013; Sperandio and Nguyen, 2012). A small proportion of *stx* gene positive isolates, with PCR, has potentially been found pathogenic to humans in United States of America(Bosilevac and Koohmaraie, 2011).

3. MATERIALS AND METHODS

3.1.Study Areas

This work was carried out from August 2017 to end of January 2018 on farms from Kombolcha, Dessie and Haik under South Wollo administrative zone of Amhara Regional state. These study sites are among big towns in Amhara located in the eastern part of the region all on the way to Mekele, 381, 401 and 427 Kms respectively away to the northeast of the capital city, Addis Ababa. Global position of Kombolcha, Dessie and Haik on google earth tool (2018) are respectively indicated for a latitude and longitude of 11° 5′N 39° 44′E, 11° 8′N 39° 38’E and 11° 19’N 39°41’E (Figure 2) with an approximate elevation of 1864, 2521 and 2003 meters above sea level. These sites comprised of small, medium and large-scale sized dairy farms supplying milk and milk products to consumers in and surrounding their towns as far as Logia and Samara of Afar Regional State.

Figure 2: Location of the study area adopted from (Gebregiorgis and Tessema, 2016)

3.2.Study Population

Animals included in this study were calves under 4 months of age that were clinically affected with diarrhea. All of these calves were found suffering differently with various degrees of diarrhea, dehydration, emaciation and weakness. Moderate to severe diarrhea was observed in all infected calves. All calves showing diarrhea were sampled regardless of other clinical parameter taken under consideration. Severe case of diarrhea which ended with death was also reported during the study session. Sampling was made in all diarrheic calves below 4 month of age that didn’t take antibiotics.

123 diarrheic calves reared within 53 farms in Kombolcha, Dessie and Haik, were sampled where each sites accounted for 55, 59 and 9 calves respectively. For the sake of distinguishing risk factors, calves were classified based on farm size(head of dairy cows) as small (<5), medium (6-50) and large (>50); age (wk/s) of calves (≤1, 2-4, 4-6, and 6-12)(Yeshiwas and Fentahun, 2017), breed(local, cross and exotic) and sex. Calves were found kept with their dam or in isolated pens for the whole day. In most farms calves aged a month and more were observed being released out for grazing.

3.3.Study Design and Sampling Methodology

A cross sectional study was conducted in dairy farms from August 2017 to January 2018 and purposive sampling method was used to collect fecal samples. The study sites were selected by convenient sampling method. Fecal samples were obtained after digital stimulation of the rectal mucosa using disposable latex glove. Approximately 30gm of diarrheic feces having different types of appearances were collected from calves less than four months of age. Specimens were placed in dry, leak proof, sterile plastic tube and raw data was always recorded (date, farm name, calf id, sex, age, breed and color of diarrhea). Samples were then placed into universal ice box containing ice packs and those taken from Dessie and Haik were transported to Wollo University, school of veterinary medicine, microbiology laboratory within 3 h of collection and stored at 4°C. However, samples obtained from Kombolcha were taken to and processed at Kombolcha regional veterinary diagnostic laboratory, department of microbiology. All samples were processed within 24–48 h after reception.

3.4.Isolation and biochemical characterization of *E. coli*

Each fecal sample was streaked onto Eosin methylene blue (EMB) agar using wire loop in a laminar flow hood, and then incubated at 37 °C overnight. Those colonies showing characteristic metallic sheen appearance on EMB agar were considered as presumptive *E. coli.* Additionally, 4 to 5 colonies were then picked up and streaked on MacConkey agar to detect bacterial nature for lactose fermentation overnight at 37 °C as described by Gebregiorgis and Tessema, (2016). Both positive (rose pink) and negative (pale) colonies were then stored in nutrient broth at 4 °C for further identification by biochemical (IMViC) tests.

Those pure isolates stored in nutrient broth were further streaked on EMB agar to process large samples later for their biochemical activity. Four to five colonies were selected and confirmed to be *E. coli* by standard biochemical tests for Indole(I), methyl red(M), Voges-Proskauer(V) and citrate utilization tests(C) commonly called IMViC test. Isolates exhibited IMViC pattern of (+ + - -) respectively were considered as *E. coli* and were stored at -20 °C in nutrient broth containing 20% glycerol until use.

3.5.Antimicrobial Sensitivity Testing

Desired numbers of *E. coli* isolates were transported to Addis Ababa University, Institute of Biotechnology to detect antimicrobial resistance for eleven paper impregnated antibiotic discs. Isolates were revived on EMB agar again and colonies transferred to nutrient broth that had later evaluated using standard disc-diffusion methods on Mueller Hinton agar. Susceptibility and resistance of different antibiotics were measured *in vitro* by employing the Kirby – Bauer – disk diffusion method (Bauer *et al.*, 1959).

The turbid broth over night was swept on Mueller – Hinton Agar and antibiotic impregnated disks were applied on plates. Eleven different commercially available disks were impregnated with the following antibiotics (Oxoid Basingstoke, Hampshire, UK); Compound sulfonamides (300 µg), clindamycine (10 µg), streptomycin (10 µg), nitrofrontoin (300 µg), ciprofloxacin (5µg), chloramphenicol (30 µg), gentamycin (10 µg), neomycin (10 µg), Trimethoprim (5µg), ampicillin (10 µg) and oxytetracycline (30 µg). All the disks were gently pressed down onto the agar with sterile forceps and incubated aerobically for 18 hours at 37 ºC. The diameters of the zones of inhibition were measured using transparent ruler and result was recorded as susceptible and resistant. Interpretation was made based on the recommendations of Clinical Laboratory Standards Institute CLSI (2018) for nine antimicrobials and Tendencia (2004) manual for neomycin and clindamycine.

3.6.Virulent Gene Detection

3.6.1. DNA Extraction

A single bacterial colony grown on EMB agar (Oxoid Basingstoke, Hampshire, UK) was inoculated into nutrient broth (Oxoid Basingstoke, Hampshire, UK) and incubated at 37ºC for 18h. After

incubation, aliquot of 1.5 ml was taken from nutrient broth and transferred into sterile DNase and RNase free 2 ml eppendorf tubes. Tubes were then centrifuged at 13,000 rpm for 10 min. The bacterial pellets were lysed by boiling in 50 µl of double distilled water in a water bath at 95 ^{0}C. The lysate was centrifuged again as before and the supernatant was used directly as template for PCR(Abdelhai *et al.*, 2016).

3.6.2. Detection of Virulent Gene Sequences By PCR

Reagent optimization: DNA extracts of each *E. coli* isolates were subjected to PCR for the presence of target genes. According to the annealing temperatures of the different primers used, six PCR assays were performed. Master Mix was carried out in a 200 µl PCR tube (Himedia; India, 2017). Mixture was made with double distilled water, PCR buffer (Himedia) containing 17.5 mM $MgCl_2$, 0.35 mM of each dNTPs (Himedia; India, 2017), virulence gene-specific forward and reverse primers (Himedia; India, 2017), Taq polymerase enzyme (Himedia) and DNA template. To detect all six virulence genes the numbers of samples to be processed were primarily decided and calculation for multiple of 22 µ1 was determined. Accordingly, all well optimized reagents, mentioned above were mixed up and aliquot of master mix was made for each isolates needed. Eventually, 3 µl of DNA template were added which made a 25 µl reaction set up ready to amplify in thermal cycler.

stx_1, stx_2, eae, bfpA, hlyA and aatA were target genes of each isolates using forward/reverse primers EVS1/EVS2, EVT1/EVT2, EAE1/EAE2, BFPF/BFPR, EHECF/EHECR and EAECF/EAECR respectively. These virulent genes except *bfpA*, optimization of necessary reagents was shared with equal volume of double distilled water (16 µl), PCR buffer (2.5 µl), primer (1 µl each), mixed dNTPs (1 µl), Taq DNA polymerase enzyme (0.5 µl) and 3 µl of DNA template of each isolate. However, *bfpA* gene was amplified using the optimized conditions of 17 µl of double distilled water, 2.5 µl of PCR buffer, 1 µl of mixed dNTPs, 0.5 µl of forward primer (BFPF), 0.5 µl of reverse primer (BFPR), 0.5 µl of Taq DNA polymerase enzyme and 3 µl of DNA template.

Adjusting PCR condition: The reaction mixture containing PCR tube was then placed appropriately in a thermal cycler (Applied Biosystems StepOne™ Real-Time PCR System Thermal Cycling Block). This system classified the amplification process in to four segments, segment1 (initial denaturantion), segment2 (final denaturation, annealing and initial extension), segment3 (final

extension and segment4 (cooling stage 25^0c for 30 sec.). For this particular work 30 and above cycles were used; whereas, other segments were progressed in 1 cycle.

To detect reaction mixture for *stx*$_{1,}$ *stx*$_2$ and *bfpA* genes it was adjusted with an initial denaturation temperature of 95^0c for 3 min in a cycle, next stage for final denaturation, annealing and initial extension took 30 cycles each consisting 40 s at 95^0c, 40 s at 55^0c, 30 s at 72^0c respectively and a final extension of 1cycle for 8 min. at 72^0c. The reaction mixture for *hlyA* and *aatA* were amplified with an initial denaturation of 1 cycle for 3 min. at 95^0c; 30 cycles each consisting, 40 s at 95^0c, 1 min. at 45^0c, 1 min. s at 72^0c; and a final extension of 1 cycle for 10 min. at 72^0c. To detect *eae* gene amplification was made with an initial denaturation temperature of 95^0c for 3 min in 1 cycle, followed by 35 cycles each consisting 40 s at 95^0c, 1 min at 55^0c, 1 min at 72^0c respectively for final denaturation, annealing and initial extension. Final extension was 1 cycle for 10 min. at 72^0c.

3.6.3. Agarose Gel Electrophoresis

The PCR products (10 µL aliquots with loading dye) were resolved in 1.5% agarose gel containing 5 µL ethidium bromide in 1× TBE buffer (40 mM Tris-HCl, 20 mM Borate, 0.5mM EDTA, pH 8.3). Each well of the gel was placed with DNA ladder (100 and 1 kb plus bp) (Himedia; India, 2017) used as a molecular size marker, positive control, list of sample amplicon and negative control respectively from left to the right. The electrophoresis was carried out at 120 V for 30 minutes.

3.6.4. Gel documentation

After time for electrophoresis was over, gel was visualized and photographed under the Alliance 4.7 XD-79 System (Biored Gel Doc XR, USA), gel documentation machine, where results were recorded from each well. Amplicon size was estimated either from previous works or using Basic Local Alignment Search Tool (BLAST) deposited in GenBank that have 100% similarity to nucleotide sequences mentioned as below.

Table 1: Primer gene sequences, target gene and amplicon sizes

Primer	Nuclotide sequence(5'---3')	Amplicon size	Target gene	Reference
EVS1 EVS2	F- ATCAGTCGTCACTCACTGGT R- CTGCTGTCACAGTGACAAA	110 bp	stx_2	Selim *et al.,* (2013)
EVT1 EVT2	F- CAACACTGGATGATCTCAG R -CCCCCTCAACTGCTAATA	349 bp	stx_1	Selim *et al.,* (2013)
EAE1 EAE2	F- AAACAGGTGAAACTGTTGCC R- CTCTGCAGATTAACCTCTGC	490 bp	*eae*	Khan *et al.,*(2002)
EHECF EHECR	F- ACGATGTGGTTTATTCTGGA R- CTTCACGTCACCATACATAT	165 bp	*hlyA*	Selim *et al.,* (2013)
EAECF EAECR	F- CTGGCGAAAGACTGTATTCAT R- CAATGTATAGAAATCCGCTGTT	630 bp	*aatA*	Havt *et al.,* (2017)
BFPF BFPR	F- AATGGTGCTTGCGCTTGCTGC R- GCCGCTTTATCCAACCTGGTA	324 bp	*bfpA*	(Bakhshi *et al*., 2013)

3.7.Questionnaire Survey

Amharic version of questionnaire was distributed to all dairy farm owners to assess the general calf husbandry practices. The questionnaire broadly encompasses academic level and associated awareness base of both owner and calf attendant, intrinsic nature of calves born in the farm, overall feeding activities, calf interaction with their environment and possible therapeutic intervention while diarrheic.

3.8.Ethical approval

Ethical clearance was obtained from the Departmental Research and Ethical Committee (DRERT), Department of Biochemistry, School of Medicine, Addis Ababa University (DREC 01/17). Supportive letter to all farm owners in the study areas was given from livestock development promotion agency Kombolcha animal disease survey, investigation and diagnostic laboratory (1116/t.3109 and 1117/t.3109). At the course of sample collection the objectives of the work was explained to farm owners.

3.9.Data Management and Analysis

All questionnaire, generic data and laboratory result recording formats were designed in epiinfo (epiinfo^{TM}) version 7.2.1.0 software and raw data were entered accordingly. Available data were then exported to Microsoft Excel® 2007 where entered data to be classified, filtered and coded. This data was eventually transferred to SPSS (SPSS INC.Chicago, IL) software version 20 for appropriate statistical analysis. The prevalence of *E. coli* isolates, virulent genes and their unconditional associations with independent variables were first determined and screened using descriptive statistics analysis.

All relevant independent variables such as study area, age, housing, method and types of feeding were screened and selected for further multivariate logistic regression analysis. Thus the effect of these factors on the occurrence of gene/s bearing *E. coli* was summarized using multivariate logistic regression analysis with the method of backward stepwise process to fit the final model. Chi-square (x^2) test was applied to which effects reported statistically significant when the calculated P-value was less than 0.05.

4. RESULTS

4.1.Occurrence of *E. coli* and its association with various factors

A total of 110 from 123 (89.4%) diarrheic samples studied showed metallic sheen on EMB agar medium from which three were non lactose fermenters (pale colony on macConkey agar). On a sequential biochemical assay 10(9%) colonies that exhibited metallic sheen on EMB agar were found to be non-*E. coli* based on IMViC test. Thus, a total of 100/123 (81.3%) isolates were found to be *E. coli*. The natural tendency for susceptibility and the occurrence of *E. coli* assessed with the following factors is placed in the following (Table 2).

Table 2: Distribution and association of *E. coli* with natural factors

Factors		Number tested	*E. coli* positive	P - value
Areas	Kombolcha	55	46	0.552
	Dessie	49	46	
	Haik	9	8	
Sex	Male	64	49	0.344
	Female	59	51	
Age	≤1wk	27	22	0.143
	1<age≤4 wks	64	55	
	4-6 wks	20	15	
	6-12 wks	12	8	
Breed	Local	3	1	0.631
	Cross	8	6	
	Exotic	112	93	

Factors practiced in the farm are important for recycling of pathogen in general and pathogenic strains of *E. coli* infection of calves in particular. Accordingly, the following (Table 3) revealed the distribution of *E. coli* in accordance with some of these management related factors.

Table 3: Distribution and association of *E. coli* with management factors

Factors		E. coli positive	P. value
Calve housing	Same barn	24	0.001
	In pen	76	
Type of feeding	Milk	21	0.015
	Milk with supplement	57	
	Milk replace	22	
Method of feeding	Suckling	17	0.049
	Hand feeding	83	
First feeding of colostrums	≤ 6 hrs	58	0.673
	6 -24 hrs	37	
	>24 hrs	5	
Farm size	Large	49	0.839
	Medium	36	
	Small	15	

Due to the similar management procedures that most studied farms followed, no statistical comparisons were done for most of the farm factors and no relevant association was found to various variables. However, some of the potential risk factors mentioned (Table 2 and Table 3) are used in comparison for occurrence of *E. coli*. Almost all of the farms (except one) had knowledge for immunological importance of colostrums to whom 61.8% were responded to feed neonates within 6 hours of calving and 39.2% were practiced it within 24 hours. Among the respondents 20.3% allowed their calves freely to suckle their dam after birth and 79.7%were used hand (bottle) feeding of colostrums to which 15.4% and 84. 6% respectively were positive for *E. coli*. However, as mentioned on table 4 approximately an equal proportion of virulent gens were derived.

4.2.Antibiotic resistance patters of *E. coli* isolates

No isolate was found susceptible to all eleven antimicrobial agents. Rather this finding shows large amount of *E. coli* isolates got resistance for one or more of antimicrobial agents used. Based on this fact one isolate (1.3%) developed resistance only for clindamycine, 6 isolates (7.8%) for 2

antimicrobials, 10(13%) isolates for three, 17 isolates (22.1%) for four antimicrobials, 27 isolates (35.1%) for five and 16 *E. coli* isolates (20.8%) were resistant for six antimicrobials.

On the other hand, one isolate (H4) was susceptible to ten antimicrobial agents (only resistant to clindamycicine) and other six isolates were resistant to only sulfonamides and clindamycine and were susceptible to other nine agents. In addition to this, all *E. coli* isolates processed were susceptible to three antimicrobial agents namely; Cloramphenicol, Gentamycine and Neomycine. Seventy isolates (91%) were multidrug resistant, i.e. resistant to three or more antimicrobials. Proportion of isolate resistance for each antimicrobial drug has been summarized in the following bar chart.

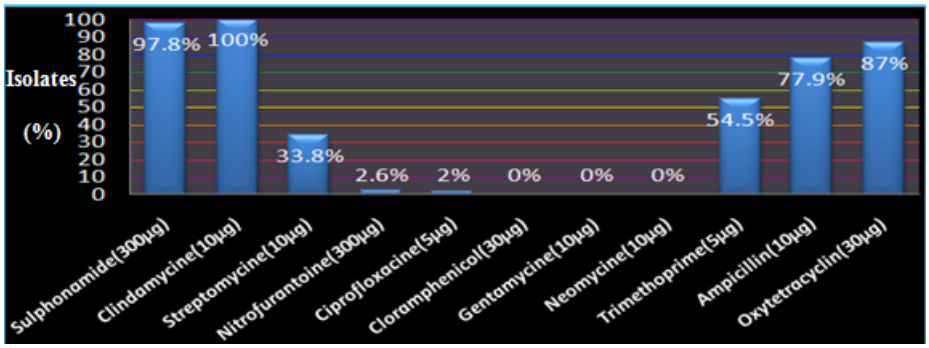

Figure 3: Antimicrobial resistance pattern of all 77 *E. coli* isolates.

Multidrug resistance was analyzed if there was any association with study areas and was confirmed to be significantly higher in Kombolcha [P= 0.019, OR= 18.600 (1.601-216.056)] and Dessie [P= 0.023, OR= 10.500 (1.393-79.130)] than Haik.

4.3.Virulence genes and derived pathogenic *E. coli* strains in diarrheic calves

Out of the total isolates 77 isolates were generally processed with PCR to detect virulent genes/strains of *E. coli*. All isolates were analyzed for 4 different virulence genes (*stx_1, stx_2, eae and aatA*) by PCR at the institute of biotechnology. Positive isolates for *stx_1, stx_2 and eae* were then further examined for *hlyA* gene and those only *eae* positive isolates were tested for *bfpA* gene. Accordingly, 23(29.9%) isolates were found to carry one or more virulent genes. Among positive isolates, 16 (69.6%) harbored a single virulent gene from which 8 of them (34.8%) carried *stx_1*, 4 (17.4%) carried *stx_2* and remaining 4 (17.4%) isolates have *eae*. No isolates were detected positive

for *bfpA* and *aatA* genes in all processed sample. On the other hand 7 isolates (30.4%) were confirmed to have two or more virulent genes on which one isolate (K22) harbored both stx_1 and stx_2 genes (4.3%), four isolates (D18, D33, D42 and D44) carried both stx_1 and *eae* genes (17.4%). Likewise, one isolate (D20) carried both stx_2 and *eae* gene (4.3%) and all stx_1, stx_2 and *hlyA* genes were detected in one *E. coli* isolate (H2).

Predisposing factors for acquiring bacteria or eliciting diarrhea to all positive virulence genes were also analyzed. Based on their relevance after analysis, the following variables were found to be relatively predicators of isolates that carried virulent genes as shown in Table 4.

Table 4: Comparison of *E. coli* isolates that carried virulent gene/s with relevant factors

Gene/s in each isolate	Factors for acquiring virulent gene/s carried *E. coli*													
	Areas			Age (wk/s)				Housing		Feed type			Feeding method	
	K	D	H	≤1	2-4	4-6	6-12	Pen	Barn	M	WS	MR	Suckling	Hand
stx_1	1	6	1	1	7	-	-	1	7	4	4	-	5	3
stx_2	1	3	-	-	4	-	-	1	3	-	4	-	-	4
eae	3	1	-	1	2	1	-	3	1	1	3	-	1	3
stx_1+stx_2	1	-	-	1	-	-	-	-	1	1	-	-	-	1
stx_1+eae	-	4	-	1	1	1	1	3	1	1	2	1	3	1
stx_2+eae	-	1	-	-	1	-	-	-	1	-	1	-	1	-
stx_1+stx_2+hlyA	-	-	1	-	-	1	-	-	1	-	-	1	1	-
Total	6	15	2	4	15	3	1	8	15	7	14	2	11	12
P- value	0.065			0.01*				0.001**		0.175			P = 0.195	

K=Kombolcha; D=Dessie; H=Haik; M= milk; WS= with supplement; MR= Milk replace; * = P ≤ 0.05; ** = P <0.01

The distribution of stx_1, stx_2, *eae* and *hlyA* genes with respect to study areas, age category, housing, types of calf feed, ways of providing colostrums and milk are presented in Table 4. Accordingly, stx_1 (n = 14) and *eae* (n = 9) together (Figure 4) constitute 74.2% of all virulent gene distribution and samples collected from Dessie were the predominant sources.

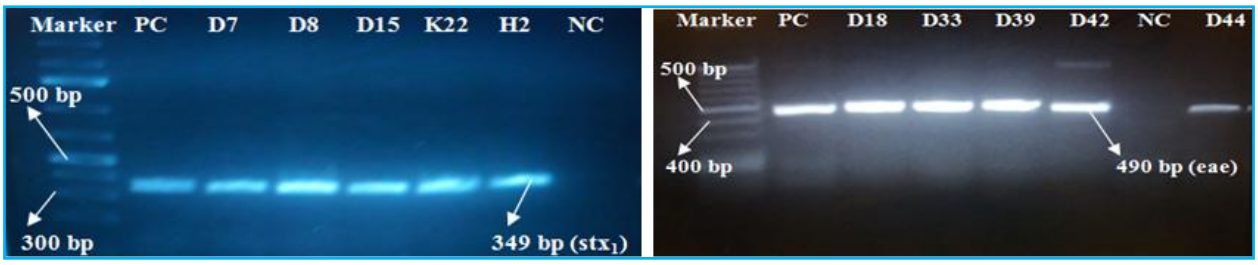

Figure 4: Agarose gel electrophoresis of amplified *stx*$_1$ (349 bp, left gel) and *eae* (490 bp, right gel) genes of 5 isolates. Where PC= positive control and NC=negative control.

On the other hand *stx*$_2$ (n=7) and *hlyA* (n=1) contribute for the remaining 25.8% of virulent genes (Figure 5) where hlyA were established from STEC. *stx*$_2$ gene (9.1%) identified less frequently than *stx*$_1$ (18.2%) among the whole isolates.

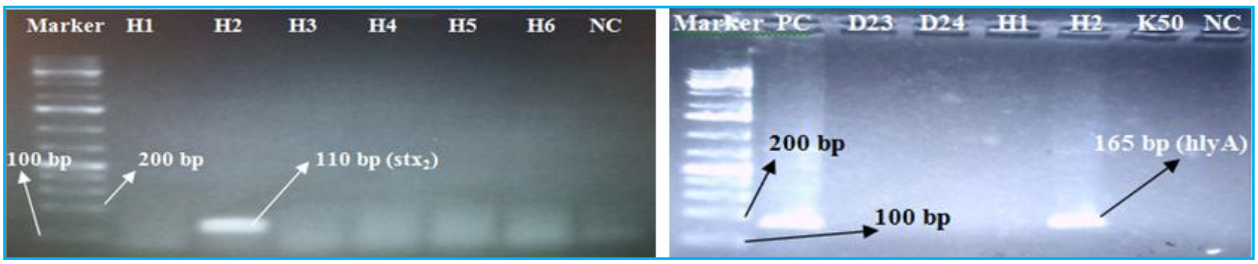

Figure 5: Agarose gel electrophoresis of amplified *stx*$_2$ (110 bp; left gel) and *hlyA* (165 bp; right) genes. Where PC= positive control and NC= negative control

On the other hand, plasmid genes *bfpA* and *aatA* were detected in none of processed samples (Figure 6). *bfpA* is investigated from 4 isolates that are previously confirmed exclusively for *eae* gene, where as all 77 samples were tested by PCR for existence of *aatA* gene.

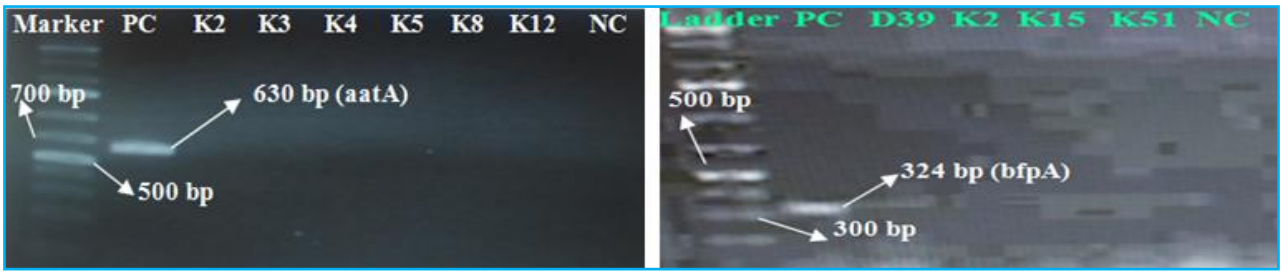

Figure 6: Agarose gel electrophoresis of amplified *aatA* (630 bp; left) and *bfpA* gene (324 bp; right) genes appeared on positive control of both gel.

Those risk factors having unconditional association mentioned above (Table 4) were further analyzed to their level of contribution under two categories as natural factors (study area and age)

and management factors (housing, feed type and type of feeding). Result is summarized in Table 5 using multivariate logistic regression analysis in the domain of 95 % of confidence interval.

Table 5: Factors contributing to virulent gene bearing *E. coli* infection and diarrhea

Factors	Category	P value	OR(95% CI)
Area	Kombolcha	0.345	2.764(0.335-22.821)
	Dessie	0.078	6.024(0.815-44.512)
	Haik	Reference category	
Age	≤ 1 wk	0.606	1.833(0.183-18.370)
	2-4 wks	0.008	20.625(2.241-189.840)
	4-8 wks	0.372	3.000(0.269-33.487)
	8-12 wks	Reference category	
Housing	In the same barn	0.001	12.217(3.475-42.951)
	In separated pen	Reference category	
Type of feeding	Milk	0.057	6.585(0.948-45.759)
	Milk and supplement	0.015	9.639(1.546-60.086)
	Milk replace	Reference category	
Feeding Method	Suckling	Reference category	
	Hand feeding	0.924	1.077(0.234 - 4.967)

As indicated in Table 5, calves aged between 2 wk to a month have significantly higher risk (p = 0.008) of acquiring virulent gene carrying *E. coli*. Meanwhile, calves living in the same barn with their dam were found to have significantly higher risk (p = 0.001). On the other hand, those calves beginning to take supplements with milk also have significant association (p = 0.015) to this bacteria. In connection with type of supplementary feed, the variation in gene acquisition by providing hay and concentrate was analyzed separately using univariate logistic regression. Interestingly those calves supplemented with concentrate revealed significantly higher [p=0.028, OR= 6.045(1.216-30.055)] risk of carrying *E. coli* with virulent genes.

In general from 23 positive isolates 14 (60.9%), carried the *E. coli* strains with a variant of stx_1, stx_2 *or* stx_1/stx_2, $stx_1/stx_2/hlyA$ genes (rendering STEC strain) and four (17.4%) were positive for only *eae*, categorized as atypical EPEC (aEPEC). The remaining five (21.7%) are entero-hemorrhagic

(EHEC) strains to which isolates with stx_1 or stx_2 genes simultaneously carried *eae*. Based on the virulent genes indicated in Table 4, the distribution of pathogenic *E. coli* strains is summarized in the following (Table 6).

Table 6: Distribution and association of pathogenic strains of *E. coli* with various factors.

Factors	Strains and their frequency				P - value
		EPEC	EHEC	STEC	
Area	Komblcha	3	-	3	0.093
	Dessie	1	5	9	
	Haik	-	-	2	
Age (wk/s)	≤ 1	1	1	2	0.004
	2 - 4	2	2	11	
	4 - 6	1	-	1	
	6 - 12	-	1	-	
Housing	The same barn	1	2	12	0.001
	Isolated pen	3	3	2	
Feed type	Milk	1	1	5	0.049
	With supplement	3	3	8	
	Milk replace	-	1	1	
Feeding type	Suckle	1	3	6	0.328
	Hand	3	2	8	

4.4.Virulent genes and associations with antimicrobial resistance

Out of the 23 isolates that carried virulent genes, twenty two (95.7%) developed multidrug resistance. These isolates are completely resistant to sulphonamides and clindamycine. On the other extreme, isolates revealed entire susceptibility to four antimicrobial agents (Nitrofurantoin, Cloramphenicol, Gentamycine and Neomycin) which is summarized in Table 7.

Table 7: Virulent gene carried isolates and their resistance to spectrum of antimicrobials.

Virulent gene/s in each isolate	Strains	Resistant antimicrobials to isolates										
		SA	Clin	Strep	Nit	Cip	CA	Gen	Neo	Trm	Amp	OTC
stx_1	STEC	8	8	1	-	1	-	-	-	4	6	6
stx_2	STEC	4	4	3	-	-	-	-	-	4	4	4
eae	EPEC	4	4	1	-	-	-	-	-	1	4	4
stx_1+stx_2	STEC	1	1	-	-	-	-	-	-	-	1	1
stx_1+eae	EHEC	4	4	4	-	-	-	-	-	4	4	4
stx_2+eae	EHEC	1	1	1	-	-	-	-	-	1	1	1
stx_1+stx_2+hlyA	STEC	1	1	-	-	-	-	-	-	-	1	-
Total	-	23	23	10	0	1	0	0	0	14	21	20

SA = sulphonamides, Clin = Clindamycine, Strep = Streptomycine, Nit = Nitrofurantoin, Cip = Ciprofloxacine, CA = Cloramphenicol, Gen = Gentamycine, Neo = Neomycine, Trm = trimethoprime, Amp = Ampicillin and OTC = oxytetracycline.

Based on the information depicted in Table 7, resistance isolates showed full resistance to full sensitivity to antimicrobials. All virulent gene carrying isolates were completely resistant (100%) to clindamycine and sulphonamides followed by 91.3% to Ampicillin, 87% to Oxy tetracycline, 60.9% to Trimethoprime, 43.5% to Streptomycine, 4.3% to Ciprofloxacine and sensitive to the remaining four antibiotics. We classified strains that showed resistance to three or more antimicrobials as MDR. On the other hand, all EPEC strains are entirely resistant to sulfonamide, clindamycine, ampicillin and oxytetracyclin whereas EHEC strains developed complete resistance for 6 antimicrobials namely; sulphoneamides, clindamycine, streptomycine, trimethoprime, ampicillin and oxyteteracycline. Additionally all STEC strains are resistant to less number of antimicrobials namely for clindamycine and sulfonamides.

5. DISCUSSION

Diarrheagenic *E. coli* is incriminated as an important cause of economic loss and hinders sustainable development of the dairy sector; as a result detailed studies of the virulence factors produced by *E. coli* strains in farm animals are needed. Of the 123 fecal samples collected from the diarrheic calves 100 (81.3%) were confirmed positive for conventional biochemical tests. Biochemically confirmed isolates were proportionally higher than recent works 70.7% of Yeshiwas and Fentahun (2017), 36.8% of Gebregiorgis and Tessema (2016) from Debrezeit and Kombolcha respectively from Ethiopia. Shahrani and his colleagues (2014) in Iran also reported 76.45% prevalence prior to molecular confirmation. Isolation rate of *E. coli* were not statistically significant among age, sex, breed, study areas (Table 2).

Similarly, management factors including farm size and time for onset of colostrums feeding (Table 3) did not associate with *E. coli* positivity. The knowledge about the immunological importance of colostrums among farm owners might be the reason for similar types of management strategies in those farms. It was also noted that isolation rate of *E. coli* had statistically significant association with management factors including, calve housing ($p = 0.001$), type of feed ($p = 0.015$) and method of feeding ($p = 0.049$). The difference in *E. coli* prevalence in various areas might be due to lack of hygiene, overcrowding, lack of post milking teat dipping, lack of effective preventive measures, differences in sample size and also diagnostic techniques used for the detection of *E. coli* (Hossain *et al.*, 2013).

Overall, 23 (29.9%) calves (one isolate from each calf) carried at least one of the tested virulence genes giving the type of strain EPEC, EHEC or STEC. This is in agreement with the work of Herrera Luna *et al.*, (2009) reported as 28.9% for similar virulent genes. This finding is greater than the work (6.9%) of Badouei and his colleagues (2014). 43.3% of these virulent genes were detected from dairy farms of Hawassa, Ethiopia among 29 isolates (Tsedale *et al.*, 2018).

The stx_1 is an important virulent gene detected in this study most importantly incriminated as diarrheagenic in both calves and children with stx_2. In this finding, fourteen stx_1 genes (45.2% of all confirmed genes and 18.2% of all isolates) were found either alone per isolate or in combination with other virulent genes of an isolate. Of these, eight stx_1 genes were found exclusively in eight isolates, one combined with stx_2, four in combination with intimin gene (*eae*) and the other one was

found in an isolate as a component of triple gene with *stx*$_2$ and *hlyA*. Hashish *et al.,* (2016) found this gene with frequency of 86.67% and 41.3% also reported from De Moura *et al.,* (2012) in Brazil to which both are much higher than our finding.

The *stx*$_2$ is another important virulent gene in STEC and EHEC strains to which four isolates (constitutes 12.9% of detected genes) carried it exclusively. Co carriage of various virulent genes are common in *E. coli,* for which an isolate was positive for *stx*$_2$ and *stx*$_1$ (3.2%) in this finding, a single *stx*$_2$ gene with *eae* (3.2%) and as mentioned above this gene is also a component of triple gene carried in one isolate with *stx*$_1$ and *hlyA* (3.2%). Prevalence of 26.67% was recorded by Hashish *et al.*, (2016). Tahamtan and his colleagues (2010) mentioned that strains possessing only *stx*$_2$ are potentially more virulent than strains harboring *stx*1 or even than those that carried both *stx*$_1$ and *stx*$_2$ in human. In connection with this, Stx$_2$ has been found to be approximately 400 times more toxic than did Stx$_1$.

The frequency of *stx*$_1$ in this study is found higher than other detected virulent genes among all of the study sites. The predominant occurrence of *stx*$_1$ was also detected in Egypt(Hashish *et al.*, 2016), Brazil(De Moura *et al.*, 2012) and Iran(Taghadosi *et al.*, 2018). Additionally, isolates form Uruguay were found carrying no *stx*$_2$ but 4.8% of *stx*$_1$ (Umpierrez *et al.,* 2017). This finding and recent works in Ethiopia also revealed higher prevalence of *stx*$_1$ over *stx*$_2$ (Amanuel *et al.*, 2018; Tsedale *et al.*, 2018). Perhaps this might be due to similarities of season of sample collection. In contrast, a finding from Iran showed that STEC harboring *stx*$_2$ isolate was significantly more (53.42%) than harboring *stx*$_1$ (10.27%) (Tahamtan *et al.*, 2010). Similarly, 30% *stx*$_2$ harboring isolates were reported which is higher than 10% *stx*$_1$ gene (Dastmalchi and Ayremlou, 2012) from Vietnam (Nguyen *et al.*, 2011). Emphasis was given in geographical area and season of sampling as important factors in epidemiology of STEC and EHEC in animals (Badouei *et al.*, 2014).

Isolates that carried *stx*$_1$ and *stx*$_2$ genes in this particular work are either of STEC or EHEC strains. STEC has been implicated as an etiological factor of calf diarrhea(Sandhu and Gyles, 2002) and such animals form a principle reservoir of STEC that is pathogenic for humans(Badouei *et al.*, 2014). Infections with STEC have been described in a wide range of domestic and wild animal species, but the natural pathogenic role has been demonstrated only in weaning pigs, young calves, and dogs. In addition, in humans, STEC/EHEC strains are considered as major cause of HC and HUS worldwide, acquired as secondary route of infection through contact with carrier

animals(Badouei *et al.*, 2014). The gene belonging to strains detected from animals showed more expression of protein toxin than human samples, hence the strain within animal origin maintain the characteristic and are more cytotoxic than the gene from human origin (Tahamtan *et al.*, 2010).

In most research out puts, EHEC strains reportedly unified with STEC strains. Isolates that harbored both *eae* with *stx*'s genes mentioned in literatures are categorized as EHEC and is used for comparison in this discussion. In almost all works, STEC strains have higher prevalence than EHEC. For instance, in a study in Vietnam on diarrheic calves, 51.3% of their samples were positive for all *stx* genes of which 44.6% were STEC and 6.7% were EHEC (Nguyen *et al.*, 2011). STEC found in this study (18.2%) is higher than the works of Dastmalchi and Ayremlou, (2012) Umpierrez *et al.,* (2017), and Badouei *et al., (*2014) having respective prevalence of 11.8%, 0.83% and 3.4% but lower than 44.6% reported by Nguyen *et al.,* (2011) and 38.6% by Amanuel *et al.*, (2018).

On the other hand, EHEC strain prevalence in this study (6.5%) is higher than that of Umpierrez *et al.,* (2017) and Badouei *et al.,* (2014) having equal prevalence of 0.7%. EHEC strains in this finding are virtually in agreement with works of Dastmalchi and Ayremlou, (2012), Badouei *et al., (*2014), Niguyen *et al.,* (2011) and Amanuel *et al*., (2018). Other than diarrheic calves, rectal swab samples taken from apparently healthy cattle in Bangladesh also showed 43.33% STEC prevalence (Hassan *et al.,* 2017). The discrepancy may likely be due to geographical and climatic conditions and differences in the natural intestinal flora present in animal's gastrointestinal tract (Taghadosi *et al.*, 2018; Ownagh *et al.*, 2011).

Our finding on *eae* revealed the second abundantly confirmed gene (11.7%) next to stx_1 from nine isolates. Four isolates exclusively carried *eae* and remaining five isolates co existed with *stx* genes given *E. coli* strains EPEC and EHEC respectively. The remaining five amplified products of this gene were obtained in combination with stx_1 and stx_2 to which four of them were with stx_1 (12.9%) and a single *eae* gene was co-carried with stx_2 in an isolate (3.2%). The association of *eae* and *stx* genes was higher among diarrheic strains than healthy strains where the associations and the occurrence of diseases in calves were not definitive. Up to 70% of *eae* was also reported in dairy herds from USA(Lambertini *et al.*, 2015). Our finding is higher than 1.3% (Picco *et al.*, 2015), 2.1% (Umpierrez *et al.*, 2017) and 2% (Rehman *et al.*, 2014) from Argentina, Uruguay and Kashmir respectively. However, our finding (11.7%) is roughly in agreement with (16.4%) a report by

Tsedale *et al.,* (2018) but extremely higher prevalence of *eae* (60.3%) was obtained from Brazil by De Moura *et al.,* (2012).

Although EPEC are considered to induce diarrhea in different animal species and calves, very little information on the importance of EPEC in neonate ruminants is available (Badouei *et al.,* 2014). Higher prevalence of EPEC (14%) in Ethiopia was reported by Amanuel *et al.,* (2018) compared to ours (5.2%). But this finding is higher than the works of Badouei *et al., (*2014) and Nguyen *et al.,* (2011) having 2.6% and 2.1% respective prevalence from Iran and Vietnam. No logical specification for the variation of this result can be mentioned which covariates in response to other influential factors.

Tsedale and her co-workers (2018) investigated the *hlyA* gene on Hawassa dairy farms and confirmed that none of the isolates had this gene. In our finding, only one unique isolate taken from Haik co-carried it with stx_1 and stx_2 that accounted to 5.3% of STEC strains. Similar result was documented (5.9%) from India(Rehman *et al.,* 2014). This gene was also found upto 2.5% (Taghadosi *et al.,* 2018) and 60% in Iran(Dastmalchi and Ayremlou, 2012). An isolate carrying identical genes with stx_1 and stx_2 was also reported from human female urine(Selim *et al.,* 2013) and isolates with this genetic profile (stx_1 stx_2 *hlyA*) in calves from Iran was reported to be the most prevalent in calves(Dastmalchi and Ayremlou, 2012). This variation might be related with the discrepancy in prevalence of STEC and EHEC.

However, *bfpA* and *aatA* genes were not amplified entirely from the whole processed isolates. Published documents and relevant alternative were assessed on status of *bfpA* and *aatA* in typical EPEC and EAEC respectively from diarrheic calves. However, little was found about *bfpA* to which no isolates carried it from works in Sweden (De Verdier *et al.,* 2012) and Ethiopia (Tsedale *et al.,* 2018). Typical EPEC have not been found in documented works viewed on animals, perhaps humans are reservoir for these organisms.

No documents were found to explain about *aatA* in calves except recent works confirmed in three isolates (4.5%) from Hawassa (Tsedale *et al.,* 2018). However, *aatA* is found in various works of *E. coli* isolated from extra calve diarrhea. For instance 3.4% from human food sources and irrigation water(Aijuka *et al.,* 2018), 12.5% from malnourished stool sample of children(Havt *et al.,* 2017) and 35.4% from UPEC in human(Nazemi *et al.,* 2011).

hlyA, bfpA and *aatA* detected above are plasmid genes that have been found in none of the isolates (except one for *hlyA*) in this work. Such plasmid originated pathogenic genes can exit from the plasmid and re-locate in another place in the genome hence difficult to extrapolate the result and even no guarantee to specificity its existence (Nazemi *et al.*, 2011). Therefore, use of plasmid genes as indicative for pathogenic *E. coli* prevalence has its own limitation such as, its variable gene content, it may get lost on sub-culture and the plasmid may transfer and be detected in entirely unrelated bacteria which do not actually cause diarrhea(Chattaway *et al.*, 2011).

The associations of various risk factors with *E. coli* virulent gene/s that cause diarrhea on calves were further assessed. Among twenty three isolates with virulent gene/s, fifteen (65.2%) were confirmed from Dessie but no statistical association (P = 0.078) was found with study area. On the other hand, calves of age two weeks to a month revealed significant association (P = 0.008) with detected virulent genes. Calves with age of less than 4 weeks were reported to have significantly higher level of STEC (Kuyucuoglu *et al.,* 2011). Our work is different from most published works to which significantly higher prevalence was reported in age group of less than a week.

Management factor indicates strong risk association for calves living in the same barn with their dam (P = 0.001) and those calves that begun supplement feed (P = 0.015). Separate analysis to distinguish the variation in those genes carrying *E. coli* acquisition by supplement feeding revealed that providing concentrate shows significantly higher association(P = 0.028) than providing hay did. Negligence of keeping hygiene from environmental wastes while preparing concentrate feed was observed and perhaps predispose in acquiring pathogenic *E. coli.* In the covered farms, calves began supplement feed after a week of their age. The higher prevalence in virulent genes for concentrate fed calves with age between 2 – 4 weeks might be informative in the occurrence of diarrhea caused by *E. coli* due to poor hygiene.

The higher predictive value of housing in the same barn (mentioned above) also further strengthens the prediction that the source of virulent gene carrying pathogen was from environmental contaminants. From this work, those calves supplemented with contaminated concentrate in their age of 2 – 4 weeks that became diarrheic were believed to get large dose of the virulent genes preceding complete maturation of their immunity. Most works reported for calves younger than one week for *E. coli* susceptibility is not a case in this finding. This might be perhaps due to owner understanding of undergoing colostrum before calves obtain contaminated supplement. The calves

receiving a sufficient dose of colostrum had the highest odds of staying healthy(Kolenda *et al.*, 2015).

In general, our recent finding has large differences in the prevalence of detected virulence genes for varying STEC, EHEC and EPEC strains from other studies. This could be related to differences in farm hygiene, the type of samples tested, number of samples, method of sampling, experimental methodology, geographical area and climate differences in the areas where the samples were collected, which would have differed between each study.

Results of the antibiotic resistance profiling of the isolates harvested from diarrheic calves in the current study revealed that all of the *E. coli* isolates were resistant to one or more drugs. In the context of Dessie, Kombolcha and Haik for many years antibiotic is randomly used for the treatment purpose. Geographic variations was assessed and found significantly higher resistance in isolates from Kombolcha and Dessie than Haik. The reason of the discrepancy might be due to arbitrary administration of respective antimicrobials.

The antibiogram pattern of all 77 *E. coli* isolates shown in (Figure 9) indicates that antibiotic resistance is a huge threat among all the study areas. 100% of cloramphenicol and clindamycine, 97.8% of sulphonamides, 87% of oxytetracycline, 77.9% of ampicillin, 54.5 % of trimethoprime, 33.8% of streptomycine, 2.6% of nitrofurantoin and 2% of ciprofloxacine. Higher resistance of *E. coli* isolates than our work was reported by Shahrani and his colleague (2014). These have descending resistance level of streptomycin (98.25%), tetracycline (98.09%), sulfonamides (90.31%), gentamycin (79.68%), chloramphenicol (73.8%), ampicillin (71.11%), trimethoprim (62.22%) ciprofloxacin (60.31%) and lowest resistance for nitrofurantoin (23.96%).

Those detected virulence gene/s carrying isolates (23) are given in Table 4. All virulent genes are involved in isolates that have resistance to one or more antibiotics in the recent finding. All virulent gene carrying isolates were 100% resistant to clindamycine and sulphonamides followed by 91.3% to Ampicillin, 87% to Oxy tetracycline, 60.9% to trimethoprime, 43.5% to streptomycine and 4.3% to ciprofloxacine. However, isolates are found sensitive to nitrofrantoin, cloramphenicol, gentamicine and neomycine nearly similar with other works (Chirila *et al.*, 2017; Abubaker *et al.*, 2015; Pizarro *et al.*, 2013). Unlike to this finding isolates were found to have high sensitivity to Ampicillin and Tetracycline (Abubaker *et al.*, 2015; Pizarro *et al.*, 2013). Young animals show a higher prevalence of resistant fecal *Escherichia coli* for ampicillin than older calves on the same

farm. This is reported as carriage of ampicillin resistant *E. coli* by young calves has been shown to decline with calf age (Hoyle *et al.*, 2005).

From the most common resistance phenotypes of this work 95.7% isolates have developed MDR. Significant increase in MDR was shown between years of 1980s with those of 1990s and 2000s for tetracycline, sulfonamide and Streptomycine in young animals. Congruent to this, the most common resistance genes found were those encoding for tetracycline, sulphonamide, and streptomycine resistance. The *stx* genes bearing isolates of young animal were found with higher proportion of resistance gene together such as tetracycline resistant genes (*tetA, tetB* and *tetC*), genes responsible for resistance to sulfonamides (*sul1*) and trimethoprim (*dfrA1*). These have markedly increased in recent years(Chirila *et al.*, 2017). Genes like *aadA1* and *CITM* are also the most common antibiotic resistance genes that encode resistance to streptomycin and ampicillin in the diarrheic calves(Shahrani *et al.*, 2014). Likewise, *E. coli* strains carried *stx* genes especially EHEC in our finding (Table 6) developed entire resistance to the aforementioned drugs, i.e, sulfonamides, streptomycine, trimethoprime, ampicillin and oxytetracycline (derivative of tetracycline).

De Verdier and his co-worker (2012) indicated possible factor in a linkage between resistance genes and genes conferring selective advantage to colonize the intestinal lumen of calves. Streptomycin - sulfonamide - tetracyclines have a selective advantage to colonize the intestine of calves given a dietary milk supplement also in absence of antimicrobials. Notably, in the present study all of MDR isolates had streptomycin - sulfonamide – tetracycline in their phenotype. Additionally, antimicrobial residues taken within colostrum or milk from treated cow in the dry period could select for resistance in the enteric flora of calves. Epidemiology of antimicrobial resistance reportedly occurred through HGT and chromosomal mutation. Both resistance and virulence genes transferred together but HGT was found more effective than chromosomal mutation did(Giedraitienė *et al.*, 2011).

Antimicrobials being added to calf milk replacer (growth promotor and appetizer), in order to combat the stresses faced (prophylaxis) contributes for resistance(Chirila *et al.*, 2017). In calves a high occurrence of resistance can be anticipated since a large proportion of the animals are probably treated with antimicrobials (De Verdier *et al.*, 2012). Antibiotic prescription preference among veterinarians and antibiotic availability might have a role in development of resistance.

Antibiotic resistance is not limited to fecal *E. coli*. The dominant type of resistance in various food sources was found to ampicillin (13.3%), tetracycline (12.6%), streptomycin (8%)(Rasheed *et al.*, 2014). The *E. coli* isolates from harvested rainwater in one study also revealed resistance to Tetracyclines (51%), Ampicillin (50%) and Streptomycin (40%)(Malema *et al.*, 2018). Occurance of resistants isolates in the environment revealed that the direct or indirect contamination with resistant gene carried *E. coli* strain developed through continious exposure to the respective antimicrobials.

6. CONCLUSION AND RECOMMENDATIONS

This study characterized the virulence gene factors harbored by *E. coli* isolated from calf diarrhea and indicates that *E. coli* plays a role in calf diarrhea younger than a month of age. The higher cumulative occurrence of zoonotic STEC and EHEC strains implies that calves are an important reservoir for potential hazard towards farm animals and humans. This finding is therefore important for public health and preventive veterinary medicine where emergency cautions are necessary to decrease the incidence posed by those strains. Ways of feed preparation and low hygienic condition in visited dairy farms were full of ignorance which is assumed to be the major means of media for inter transmission of various pathogenic micro organisms in the farm environment. Taking into consideration the fact that the period and the scope of our study were limited further investigation should be done in the future. The wide spread antimicrobial resistance of enteric *E. coli* from diarrheic calves in this finding has been recognized as a huge threat which might make our future difficult in the treatment of such infection. Even though, antimicrobial resistance is an emerging worldwide problem in human and veterinary medicine, both in developed and developing countries, frequent and improper use of antimicrobials were observed in our study areas.

Based on the gaps observed and conclusion we made above the following recommendations, trusted to be constructive for concerned authorities, are forwarded;

- Further study should be made on large sample covering vast area.
- Other virulence genes of *E. coli* should be investigated.
- Disinfect farms and feed preparation should be in a hygienic way.
- Investigate the distribution in several animal species and human.
- Other diarheagenic pathogen should be studied simultaneously.
- Memorandum of understanding on zoonotic pathogen should be given to farm owners.
- Use active drugs like nitrofrantoin, cloramphenicol, gentamycine and Neomycin.
- Antimicrobial resistant gene should be genotyped.
- Regular antimicrobial susceptibility surveillance should be accustomed.

7. STRENGTH AND LIMITATIONS OF THE STUDY

7.1.Strength of the study

- It is the first attempt for the molecular diagnosis in the region.
- This work tried to cover the distribution of those virulent genes in two ecological areas; highland and midlands areas.
- It opens a way to the next elaborated research in the field.
- These findings would be important to formulate prevention programs and effective therapies for calf diarrhea caused by pathogenic *E. coli*.

7.2.Limitation of the study

- Unable to study other virulence genes.
- Resistant genes to those antimicrobials were not included.
- The etiology of diarrhea in this study was not clarified, since other infectious agents than *E. coli* were not searched for.
- Case control study not included.

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ANNEX 1: GENERIC DATA RECORD SHEET FORMAT

Date----------Farm ----------Enumerator ----------

No.	Calf ID	Age(week)	Sex	Breed	Clinical sign	Type of diarrhea
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						
25						
26						
27						
28						
29						
30						

ANNEX 2: PLATE ISOLATION AND BIOCHEMICAL TEST RESULT RECORD SHEET

Sample ----------Place----------Enumerator ---------

No.	Farm/site	Calf ID	Age (wk/s)	Sex	Mac (pink/Pal)	EMB (MS/NMS)	IMVCi Tests			
							I	MR	Vp	C
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										
13										
14										
15										
16										
17										
18										
19										
20										
21										

KEYS: Mac = MaCconkey agar, EMB = Eosinemethylenblueagar, I = Indole , MR = methyl Red, Vp = Vagouseprouskure, Ci = citrate.

ANNEX 3: ANTIBIOTIC SUSCEPTIBILITY TEST PROCEDURE

- E. coli isolates sub cultured onto Eosine methylene blue agar
- Incubated at 37 ^{o}C for 24 hrs.
- From a pure bacterial culture colonies were taken with a wire loop
- Colonies transfered to test tubes contained 5 ml of nutrient broth.
- Broth Incubated at 37°C for 18 hrs
- A sterile cotton swab dipped into the standardized bacterial suspension
- Agar inoculated by streaking with the swab containing the inoculums
- Plate rotated by 90° and repeated rubbing procedure two times for an even distribution of the inoculum.
- Surface of the medium allowed to dry for 3-5 minutes to access optimal moisture.
- Using sterile forceps, antibiotic disk placed on the surface of the inoculated and dried plate and pressed it down to ensure complete contact between the disk and the agar surface
- Plates incubated in an inverted position at 37°C
- Zone of inhibition measured using millimeter ruler after 24 hours.

Source; (Tendencia, 2004)

ANNEX 4: ANTIBIOTIC SUSCEPTIBILITY RESULT RECORDING SHEET

	code 1		Code2		Code3		Code4	
Abcs	Zone diametre	category	Zone diametre	category	Zone diametre	category	Zone diametre	category
S3-300								
CLN-10								
S-25								
NIT-300								
CPR-5								
C-30								
GEN-10								
NEO-10								
W-5								
AMP-10								
OT-30								

ANNEX 5: BIOCHEMICAL TEST PROCEDURE

Indole test

- Trypton broth(Himedia) prepared according to manufacturers prescription
- Autoclaved at 121^0c for 15 minutes
- Poured 2 ml of broth to test tubes
- *E. coli* isolates inoculated
- It has incubated at 37^0c for 48 hrs
- 1 ml of KOVAC's added and shaken gently
- Allowed to stand for 1-2 minutes
- Observe formation of cherry red ring at the top.

Methyl red test

- MR – VP broth prepared
- Inoculate *E. coli* and incubate at 37^0c for 48 hrs
- PH indicator Methyl red was added
- Red color observed for positive result and orange color fro negative one

Voges-proskauer (Vp)

- MR – VP broth prepared(Himedia)
- Inoculate *E. coli* and incubate at 37^0c for 48 hrs
- Alpha naphtol added followed by potassium hydroxide
- Color change observed red(positive) or yellow(negative)

Citrate utilization test

- Citrate slant prepared (Himedia)
- Inoculate *E. coli* and incubate at 37^0c for 48 hrs
- Test tubes observed for color change from green to blue

Source; (Hemraj *et al.*, 2013)

ANNEX 6: BREAKPOINTS OF ANTIMICROBIAL DRUGS USED

Antimicrobials			Break points		
Name	Drug code	Disc content(µg)	Susceptible	Intermediate	Resistant
Compound sulfonamide	S3	300	≥ 17	13–16	≤ 12
Clindamycine	CLN	10	≥ 21	15-20	≤ 14
Streptomycin	S	25	≥ 15	12–14	≤ 11
Nitrofurantoin	NIT	300	≥ 17	15–16	≤ 14
Ciprofloxacin	CPR	5	≥ 21	16–20	≤ 15
Cloramphenicol	C	30	≥ 18	13–17	≤ 12
Gentamycine	GEN	10	≥ 15	13–14	≤ 12
Neomycin	NEO	30	≥17	13-16	≤12
Trimetoprime	W	5	≥ 16	11–15	≤ 10
Ampicilline	AMP	10	≥ 17	14–16	≤ 13
Oxytetracycline	OT	30	≥15	12–14	≤11

Sources, (Clsi, 2018) and (Tendencia, 2004)

ANNEX 7: PHOTOS FOR *E. COLI* ISOLATES IN CULTURE, BIOCHEMICAL AND SENSITIVITY TESTS

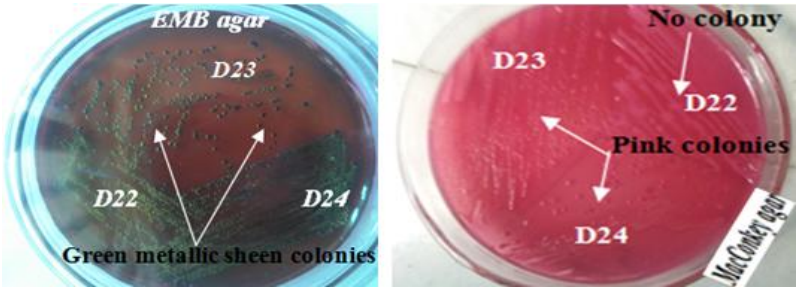

Figure 7: Pattern of E. coli colony on EMB and MacConkey agar respectively for 3 isolates

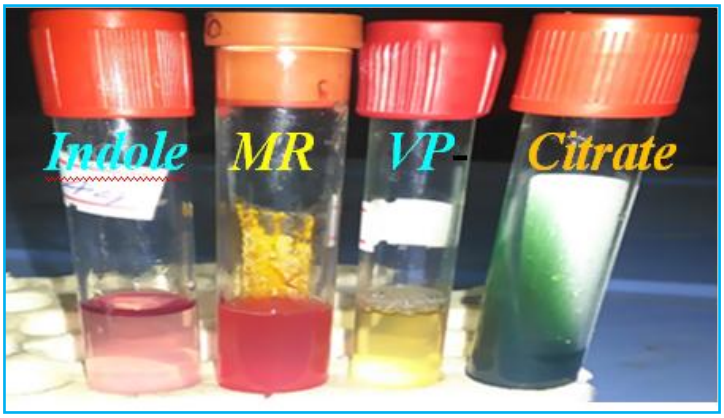

Figure 8: IMViC test showing positive for *E. coli*

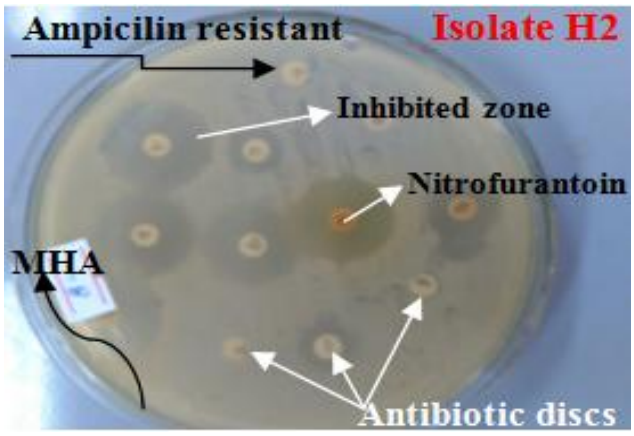

Figure 9: Disc diffusion test for E. coli isolate (H2).

ANNEX 8: VIRULENT GENE RECORDING SHEET

Isolate code	Virulence genes					
	stx_1	stx_2	eae	bfpA	hlyA	aatA

ANNEX 9: CONSENT FORM (ENGLISH VERSION)

Title: Molecular genotyping of pathogenic escherichia coli strains isolated from diarrheic calf in Dessie and Kombolcha districts, South Wollo, Ethiopia.

My name is Fentaw Hussen and I am 2nd year MSc student at Addis Ababa University, School of medicine, Department of biochemistry. I am going to molecularly characterize pathogenic strains of *E.coli* from diarrheic calves. My study needs calves younger than 4 wks that didn't receive antimicrobials. It is my pleasure while including your farm in my study. The study has neither risk on your calve nor threatens your farm at large. If you allow your farm to be one of the study destinations, you will be requested to take 10 ml of diarrheic sample directly from rectum. You have the right to mariginalize your farm from the study at any time during the course of the study. You will not get any additional benefits by allowing your farm in this study but the findings of this study will help to develop immunotherapeutic interventions for the disease, to know level of drug resistance of pathogen to local antimicrobials and also used to know distribution of *E.coli* in the study area. I assure you that any information collected from your farm for the purpose of the study will be kept confidential at all times.

For participation into the study

I have read, or it was explained to me, the information concerning this study and I understand what will be required of me if calves in my farm take part in the study. I know that my farm being in this study is voluntary and I realize that at any time I can neutralize it from this study without giving a reason and without affecting health of my calves.

_____	_____	_____
Name of dairy farm owner	Signature	Date
_____	_____	_____
Name of investigator	Signature	Date

If you have any question to the investigator please contact with the following address.

Name: Dr. Fentaw Hussein cell phone: +251920216987

ANNEX 10: የስምምነት ቅጽ (AMHARIC VERSION CONSENT SHEET)

የጥናቱ ርዕስ: ተቅማጥ አምጭ ኢስቸሪችያኮላይ የተባሉ የጥጆች ደቂቅ ተህዋስያንን በሀብለበራሂ ምርምር መለየት፡፡

ስሜ ዶ/ር ፈንታዉ ሁሴን ይባላል፡፡ የአድስ አበባ ዩኒቨርስቲ የሁለተኛ አመት የሁለተኛ ድግሪ ተማሪ ስሆን ለጥጆች የተቅማጥ በሽታ አምጭ ምርምር ሊሆኑ የሚችሉ ኢስቸርችያኮላይ የተሰኙ ደቂቅ ተህዋስያን ላይ መስራት አቅጃለሁ፡፡ እናም የእርሰዎን የወተት ላም ማዕከል በዚህ ጥናት ላይ ለማካተት እፈልጋለሁ፡፡ለማሳተፍ ፈቃደኛ ከሆኑ 10 ሚሊ ሊትር የጥጆች የተቅማጥ ፈሳሽ ናሙና በመውሰድ ምርመራው የሚካሄድ ሲሆን ናሙናው ሲወሰድ በጥጆችዎም ላይ ይሁን በድርጅተዎ ላይ የሚያመጣው ምንም አይነት ችግር አይኖርም፡፡ ድረጅተዎ በጥናቱ ላይ እንድሳተፍ ይሁንታወ በሙሉ ፍቃደኝነት መሆን ይኖርበታል፡፡ እንዲሁም ከጥናቱ በማንኛውም ስዓት የማቋረጥ መብትዎ የተጠበቀ ነው፡፡በዚህ ጥናት ላይ በመሳተፍዎ የተለየ ጥቅም አይኖርም ነገር ግን ጥናቱ በሽታውን ለማከም የሚያስችል ክትባት በማዘጋጀት ለመከላከል ያግዛል፤ ደቂቅ ተህዋስያኑ መድሃኒት የመላመድ ደረጃቸው ይታይበታል እንድሁም የስርጭታቸው መጠን በሞቃትና ቀዝቃዛ አካባቢ ላይ ያለው ልዩነት ይገመገምበታል፡፡ ለዚህ ጥናት አገልግሎት የተሰበሰቡ የእርስዎን ደርጅት የተመለከቱ መረጃዎች በሙሉ በማንኛውም ጊዜ በሚስጥር የሚያዙ መሆኑን አረጋግጥልዎታለሁ፡፡

ጥናት ላይ ለመሳተፍ ስምምነት ማረጋገጫ

ሰለሚካሄደዉ ጥናት አስፈላጊው መረጃ ስለተሰጠኝ በቂ ግንዛቤ አግኝቻለሁ፡፡በተዘጋጀው መጠይቅ ላይ የምሰጣቸው መረጃዎችም ምስጢራዊነታቸው እንደሚጠበቅ ተነግሮኛል/ተገልፆልኛል/፡፡ከዚህ በተጨማሪ በጥናቱ ወቅት የመረጃወችን ምስጢራዊነት ስለመጠበቅ እንዲሁም ለጥናቱ ትብብርም አለማድረግም ይሁን በአስፈለገኝ ሰአት ድርጅቴን ከጥናቱ ማግለል እንደምችል ተገልፆልኛል፡፡ ከላይ የተጠቀሱትን ማንኛውንም አይነት ውሳኔ ብወስንም በጥጆችም ላይ ይሁን ርባታ ማእከሉ አገልግሎት ላይ ምንም አይነት ተጽዕኖ እደማያሳድር ተነግሮኛል፡፡ ስለዚህ ግልጽና በቂ መረጃ በማግኘት አስፈላጊውን መረጃና ናሙና እድወሰድ ተስማምቻለሁ፡፡

እኔ_____አስፈላጊውን መረጃና ናሙና እድወሰድ ተስማምቻለሁ፡፡

የጥናቱ ተሳታፊ ስም _____ ፊርማ/የጣት አሻራ_____ቀን_____/_____/_____ የጥናቱ ዋና አከናዋኝ ስም _____ፊርማ_____ቀን___/_____/_____ የምስክር ስም_____ፊርማ_____ቀን_____/_____/_____ ሕጋዊ ተወካይ ስም _____የጣት አሻራ/ ፊርማ_____ቀን___/_____/_____ የድርጅቱ ሀላፊ በራሱ/ የመረዳትና መፈረም የማይችል ከሆነ ሕጋዊ ወኪል ወይም ተጠሪ ይፈርማል፤ በተጨማሪ የጥናቱ ተሳታፊም ይፈርማል፡፡

የስምምነት ተቀባይ ስም_____ፊርማ_____ቀን ___/___/

የጥናቱ ተሳታፊ ማንበብና መጻፍ የማይችል ከሆነ ይህ መረጃ በሚሰጥበት ወቅት ማንበብና መጻፍ የሚችል ገለልተኛ ምስክር መረጃውን በትክክል ስለመስጠቱ ማረጋገጥ አለበት፡፡ከዚያም የጥናቱ ተሳታፊ ተገልጾለትና ፈቅዶ መፈረሙን ይህንን ማረጋገጥ ከባድ ስለሆነ መፈረሙን ለማረጋገጥ ምስክሩም ይፈርማል፡፡

የገለልተኛ ምስክር ስም _____ፊርማ_____ ቀን _____/_____/_____

በማንኛው ም ስዓት ጥያቄ ከለዎት ጥናቱን የሚያካሂደውን ሰው በሚከተለው አድራሻ ማግኘት ይችላሉ፡፡

ስም፡ ዶ/ር ፈንታው ሁሴን፤ ስልክ ቁጥር +251-920216987፡፡ በጣም አመሰግናለሁ!!!!

ANNEX 11: QUESTIONNAIRE FORMAT (ENGLISH VERSION)

1. Farm identification

Animal code: _____ farm name:_____

Owner's name _____

Address: Kebele _____

When it established _____

Sex Male ☐ Female ☐

Age: Day 0-1Weeks ☐ 2-4Weeks ☐ 5-6Weeks ☐ 6-12Weeks ☐

Breed: Local ☐ Cross ☐ Exotic ☐

2. Farm description

Owner/manager educations status

Illiterate ☐ Read and write ☐ Elementary school ☐ High school ☐

graduate ☐ professional ☐

If professional: Related to animal production ☐ Un related to animal production ☐

Herd size: Cow ☐ Male calf ☐ Heifers ☐ Female calf ☐

The farms a source of income: Primary income ☐ Secondary income ☐

Organization of farms: Family farm ☐ Partnership ☐ State ☐

3. Management data

Calf care takers(attendants): Owner(family member) ☐ Hired help ☐

Sex of attendants': Male ☐ Female ☐

Experience of calf care takers: Less than5years ☐ Greater 5years ☐

Education of calf caretaker: Elementary school ☐ High School ☐

College graduate ☐ Professional ☐

4. Perparturient care

Calving facilities & housing: Calving pen ☐ the same barn ☐

Navel treatment: Practiced ☐ not practiced ☐

5. Awareness of attendants

About importance of colostrums feeding to neonates: Yes ☐ No ☐

If yes method of feeding: Suckling ☐ Hand feeding ☐

Time of first feeding:Less than 6 hour ☐ 6-24hours ☐ Greater 24hours ☐

Duration of feeding: For 24hrs ☐ For 24hrs to 4days ☐ > 4hrs ☐

If hand feeding–source of feeding: From dam ☐ From another cow ☐

Types of feeding: Milk ☐ Milk replace ☐

6. Amount of milk/milk replaces given daily per unit of body weight

Frequency of feeding: Once perday ☐ Twice/day ☐ Three times/day ☐

Types of supplementary feed and quality perunit of body weight:

Grazing ☐ Concentrates ☐ hay ☐

Weaning age: 4-6wks of age ☐ 6-8wks of age ☐ 8-18wks of age ☐

12-16 wks of age ☐

ANNEX 12: QUESTIONAIRE FORMAT (AMHARIC VERSION)

ı. የርባታ ጣቢያዉ መለያወች

የጥጃው መለያ ቁጥር ---------- የርባታ ጣቢያው ስም----------

የባለቤት ስም----------

አድራሻ፡ ቀበሌ----------

የተቁዋቁዋመበት ዘመን----------

ጸታ ወ ☐ ሴ ☐

እድሜ(በሳምን 0 - 1☐ 2-4 ☐ 5 – 6 ☐ 6 – 12 ☐

ዝርያ፡ የሃገር ዉስጥ☐ ድቅል ☐ የውጭ ☐

2. የርባታ ጣቢያው ዝርዝር መረጃወች

የባለቤቱ የትምህርት ደረጃ፡ ያልተማረ☐ ማንበብና መጻፍ የሚችል☐1ኛ ደረጃ☐

2ኛ ደረጃ ☐ ምሩቅ ☐ የ ሰለጠነ ☐

የሰለጠነ ከሆነ፡ ከ እንስሳት ርባታ ጋር የተያያዘ ☐ ሌላ ሙያ ☐

የእንስሳት ብዛት ላም ☐ ወንድ ጥጃ ☐ ጊደር ☐ ሴት ጥጃ ☐

ከገቢ አንጻር ፡ ዋና የገቢ ምንጭ ☐ አማራጭ የገቢ ምነጭ ☐

አደረጃጀቱ በቤተሰብ ☐ በሽርክና ☐ በመንግስት ☐

3. የእነክብካቤ መረጃ

ጥጃ ተቆጣጣሪ፡ ባለቤት (የቤተሰብ አባል) ☐ ተቀጣሪ ☐

ጸታ፡ ወ ☐ ሴ ☐

ልምድ፡ ከ 5 አመት ያነሰ ☐ ከ 5 አመት በላይ ☐

ትምህርት ደረጃ፡ 1ኛ ደረጃ☐ 2ኛ ደረጃ☐ ኮሌጅ ምሩቅ ☐ ባለሙያ ☐

4. ወሊደና ክብካቤ

መውለጃና ማደጊያ፡ ልዩ ቦታ ☐ በረት ☐

መድሃኒት የወሰደ ☐ ያልወሰድ ☐

5. የተንከባካቢዎች ግንዛቤ

የእንገር ጥቅም፡ አውቃለሁ ☐ አላወቅም☐

ካወቁ ስለ አሰጣጡ፡ በማጥባት ☐ በማለብ መመገብ ☐

የሚጀመርበት ሰአት: ከ 6 ሰአት ባነሰ ☐ 6 – 24 ሰአት ☐

ለምን ያክል ጊዜ፡ ለ 24 ሰአት☐ እስከ 4 ቀን ☐ ከዛም በላይ ☐

አልበው የሚመግቡ ከሆነ፡ ከ እናት ላም☐ ከ ሌላ ☐

የምገብ አይነት፡ ወተት ☐ ሌላ☐

6. በቀን የሚሰጠው ምግብ/ በሰውነት ክብደት

በቀን ስንት ጊዜ ይሰጣሉ፡ አንድ ጊዜ ☐ ሁለት ጊዜ ☐ ሶስት ጊዜ ☐

የተጨማሪ ምግብ አይነት፡ ግጦሽ ☐ ጥራጥሬ ☐ ድረቆሽ ☐

ጡት መጣያ እድሜ(በሳምንት)፡ 4 -6 ☐ 6 – 8☐ 8 – 18 ☐ 12 – 16 ☐

ANNEX 13: DECLARATION

I declare that this research paper entitled: Molecular identification for six virulent genes of *Escherichia coli* isolated from diarrheic calves and their resistance profile to antimicrobials in selected towns of South Wollo administrative zone, Amhara, Ethiopia, 2017/2018 is my original work and has not been presented for any degree in any other university, and that all sources of materials used for the research have duly been acknowledged

Fentaw Hussen

Signature_____

Date _____